Health monitoring of adult Lost River Sucker (*Deltistes luxatus*) and Snortnose Sucker (*Chasmistes brevirostris*) in upper Klamath Lake, Oregon, April – September 2003. Joint FWS and USGS project.

J. Scott Foott
U.S. Fish & Wildlife Service
California – Nevada Fish Health Center
24411 Coleman Hatchery Road
Anderson, CA 96007
(530) 365 – 4271 , Scott_Foott@fws.gov
Fax 530 – 365 – 7150

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Background- In support of the USGS monitoring program for endangered Lost River sucker (**LRS**, *Deltistes luxatus*) and Shortnose sucker (**SNS**, *Chasmistes brevirostris*) in Upper Klamath Lake, Oregon, the California – Nevada Fish Health Center (**FHC**) provided training, supplies, initial sample effort in April 2003, and laboratory services to examine health and several blood indices of adult suckers before, during, and after adverse water quality events. Blue green algal blooms during the summer can result in high pH ($\geq$ 9.5), low dissolved oxygen levels (<2ppm), and elevated ammonia concentrations in the lake. These periods have been associated with past fish kills. In August and September 2003 dying adult suckers were observed in the lake. Both lethal and non-lethal samples were collected from 72 adults captured in the northern area of the lake (Modoc point, Pelican Bay, Harriman creek) in April, July, August, and September 2003. Internal FHC funds were used for this work that encompassed over 200 hours of labor.

Methods - Trammel nets were fished at night and captured fish were transported to in-situ live cages prior to sample collection. Some moribund fish were collected by dipnet. Fish were anesthetized with MS-222, measured for fork length and weight, the species and sex recorded, examined for external abnormalities (gill rot, hemorrhage, sores, copepod and leech infection), a swab rubbed against the first gill filament and smeared onto a glass slide and a Tryptone Yeast Extract +Salts (TYES) agar tube, and a blood sample collected from the caudal vessels with a heprinized syringe. Gill swab imprints were stained with gram-stain and examined for filamentous gram-negative bacteria (presumptive *Flavobacterium*) at 600x magnification. On 08April, kidney – spleen samples were collected for viral assay on an EPC cell line incubated for 16 days at 15°C.

The blood sample was processed immediately by expressing several drops of blood, and placing a 20 μL drop onto both a slant tube of Brain Heart Infusion agar for bacterial isolation and a microscope slide for blood smear preparation. All bacterial samples were maintained between 10 -25 °C and shipped with blue ice to the FHC within 3 days of collection. Standard microscopic and biochemical tests (such as API-20E) were used to identify isolated colonies to the genus level. Yellow or orange pigmented colonies on TYES agar were subcultured and 3 criteria used to identify the isolate as *Flavobacterium columnare* (filamentous gram- negative rod, colony retains Congo Red stain, and proteolytic for gelatin).

Blood smears were air dried 10 minutes, fixed for 10 minutes in absolute methanol, and later stained with Diff-Quick™. A differential leukocyte count was performed at 1000x magnification on the first 100 lymphocytes, thrombocytes, neutrophils, monocytes, and eosinophil / basophils observed on the smear. A Lymphocyte : Granulocyte ratio was calculated by dividing the number of lymphocyte observed by the total of neutrophils, eosinophils, and basophils.

The majority of the blood sample was centrifuged for plasma. Three 200 μL aliquots were frozen on dry ice for shipment to the FHC. It is assumed that stress-related biases in blood chemistry values occurred in many samples as the time from initial capture to sample collection was typically > 1hr. Plasma samples were stored at -70°C until assayed for chloride, glucose, total protein, and albumin. Raichem (San Diego, CA) kits were used for chloride, glucose, albumin, and ammonia assays. The

ammonia assay proved to be highly variable and no data is reported. Total protein concentration was determined by Sigma Chemical Company kit 541-2 and sodium was assayed with a flame photometer. Albumin : globulin ratio was estimated with the calculation: [total protein] – [albumin] = globular protein concentration.

Some fish were sacrificed for bacterial samples from the spleen as well as histological samples of gill, spleen, kidney, and heptopancreas fixed in modified PREFER ^{tm} fixative (Anatech Ltd, Battle Creek MI). Tissues were processed to 5 μm paraffin sections and stained with hematoxylin and eosin. Several sections were examined under a 360 nm excitation fluorescent light to distinguish autofluorescent lipopigments. The endogenous pigment (combined hemosiderin and lipopigments) of a given tissue was rated by a subjective 0, 2, 4, 6 severity scale

O None
2 Mild (1 – 9 % of specific tissue)
4 Moderate (10 –29 % of specific tissue)
6 Extensive (>30% of specific tissue)

Results & Discussion

A total of 72 fish were sampled over the period of 08April to 05September 2003 (Table1). This collection set included 32 SNS and 40 LRS. The sex breakout was 48 females and 24 males. Any fish that was non-responsive to capture was considered "sick".

Table1. Collection groups.

Case No.	Sample date	fish condition / water qlty observation	fish no.
03-49	4-8	normal appearance/normal water	1 – 20
03-98	7-16	normal appearance / mid-bloom	21 – 40
03-107	7-30	normal appearance / bloom crash	41 – 49
03-117	8-15	normal appearance / 2nd bloom	50 – 59
03-118	8-15&19	sick fish	60 & 61
03-122	8-22 & 27	sick fish	62 & 63 –64
03-123	9-2	sick / weak fish	65 – 69
03-128	9-5	sick fish	70 –72

Pathogens - The prevalence of septicemia (blood-borne bacteria) appeared to increase after the 08April sample and remained elevated for the remaining sample groups (Table 2). No consistent association with morbidity or observations of clinical signs of septicemia (e.g. petechial hemorrhage of epidermal vessels) was apparent in the dataset. Bacteria isolated included *Aeromonas hydrophilia*, *Pseudomonas fluorescence*, and *Micrococcus* sp.. The majority of the isolations were in the mesophilic motile aeromonad complex (Aeromonas – Pseudomonas =AP) which are common inhabitants of the gastrointestinal tract and skin. Some isolations may be due to surface contamination of the needle. These bacteria tend to be opportunistic pathogens as disease is often associated with stress or due in combination with multiple pathogen infections. It is quite common to isolate these

bacteria from the internal organs of both asymptomatic cultured and wild fish. In 1997, *Aeromonas hydrophilia* was also isolated from seven moribund suckers with concurrent *Lernaea* and Columnaris infections (Appendix 3). No virus was detected in a 10 fish sample from the 08April collection.

Filamentous gram-negative bacteria (presumptive *Flavobacterium columnare)* were only detected in gill swab imprints from moribund fish with gill lesions (5 of 12 moribund fish, 42% incidence). Infections were associated with obvious gill lesions (Fig.1). All gill swab samples inoculated onto TYES agar yielded abundant bacterial growth (primarily *Staphylococcus*) but no isolations of *Flavobacterium columnare.* It would appear that surface contaminates on the Columnaris lesions interfered with isolation attempts.

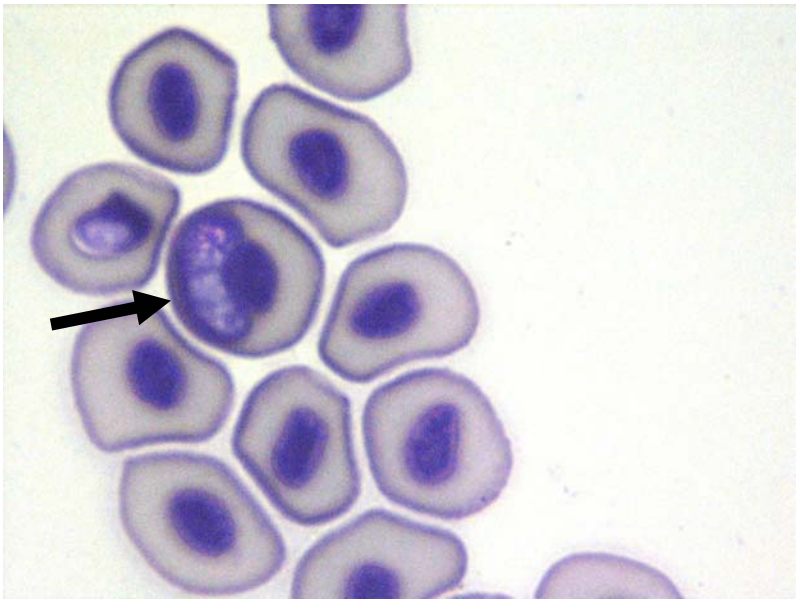
Except for the 08 April sample group, gill copepods (*Lernaea sp.*) were noted on all fish collected through September. Leeches were not observed on the external surfaces of any fish in the dataset. The blood parasite, *Haemogregrina catostomi*, was observed in 8 fish from the 08April sample group (8 / 20 = 40% prevalence) but was not associated with anemia. This coccidian parasite was found in an extremely low numbers of erythrocytes in the bloodsmears of the affected fish (Figure 2).

Table 2. Prevalence of Aeromonas / Pseudomonas (AP) ,*Micrococcus* sp. infections, total septicemia (number positive samples / total samples).

	AP	*Micrococcus*	% Septicemia
08April	2	0	2 / 20 (10%)
16July	3	0	3 / 10 (30%)
30July	3	2	5 / 10 (50%)
15August	3	1	4 / 10 (40%)
Moribund 8/15 – 9/5	8	0	8 / 12 (67%)

Figure 1. Columnaris lesion (brown eroded region, arrow) on gill from 15August collection (S. Vanderkooi,USGS).

Figure 2. Gametocyte stage of *Haemogregrina catostomi* in bloodsmear. Diff-quick stain, magnification 600x.

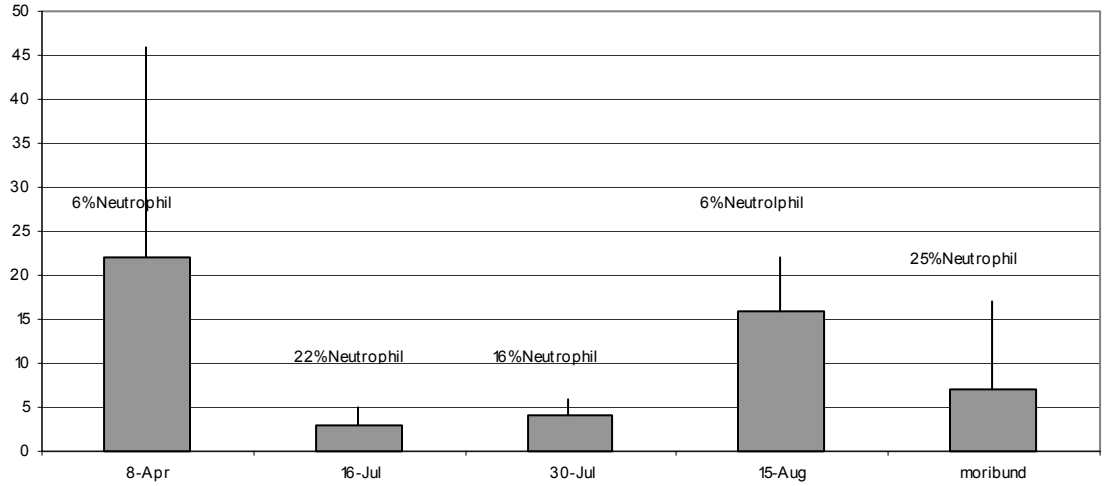


Blood samples- The relative number of neutrophils increased in bloodsmears collected on 16July, 30July, and from moribund fish (Table 3). Elevated neutrophil counts (granulocyte along with eosinophil and basophil) result in low L:G ratio values (Figure 3). Such a shift can indicate infection, tissue damage, or seasonal blood cell changes (Modra et al 1998). Similar increase in neutrophil numbers were also observed in sick fish collected in September 1997 (appendix 3). Monocyte numbers increased in some of the sick fish as a likely result of septicemia. Despite the assumed differences in water quality, the15August sample differential leukocyte count resembled the April samples. With the exception of an anemic female (fish 4, 12% hematocrit), hematocrit values ranged from 24 to 49 % in the 08April sample. The values above 30 – 35% support the assertion that handling stress was extreme for some of the fish. Epinephrine- induced splenic contraction will result in large numbers of erythrocytes put into the circulation. No further hematocrit measurements were taken due to the sampling method biases and the time required for the work.

Table 3. Mean percentage (std dev.) differential leukocyte counts.

	Lymphocyte	Thrombocyte	Neutrophil	Eosinophil	Monocyte	L:G ratio
08April n= 20	69 (19)	21 (11)	6 (7)	0.5 (1.1)	0.1 (0.3)	22 (24)
16July n= 18	42 (14)	32 (11)	22 (10)	0.4 (0.8)	0.3 (0.6)	3 (2)
30July n = 10	60 (14)	23 (10)	16 (7)	0.5 (1)	0	4 (2)
15August n = 7	82 (9)	13 (8)	6(3)	0.3 (0.8)	0	16 (6)
Moribund 8/15 – 9/5 n= 12	44 (22)	23 (11)	25(29)	2 (7)	6 (10)	7 (10)

Figure 3. Mean Lymphocyte : Granulocyte ratio (STD= bar) with mean neutrophil count listed above group LG bar.



Plasma samples - Protein concentrations were similar among the 08April, 16July, and 30July groups (Table 4 and Figure 4). Sick fish tended to be hypoproteinemic and had a similar group mean as sick fish tested in September 1997 (Appendix 3). Gill and skin damage, due to columnaris, was the likely cause of the low protein values. It is unclear why the protein values of the 15August sample group were significantly higher than all other sample groups ($P<0.05$, ANOVA). Mean albumin concentrations ranged from 1.0 to 1.8 g / dL as measured by the Bromcresol Green assay. This reagent can bind alpha proteins as well as albumin that would slightly over-estimate the actual albumin concentration (Jacobs et al. 1990). No descriptive statistics were performed on the albumin data due to the perceived bias. There was no statistical difference (ANOVA, $P > 0.5$) between the Albumin : Globulin (A:G) ratios of the 08April and moribund fish (Table 4). The loss of plasma protein, due to epidermal lesions, was a likely factor in the high variability (244% Coef. of variation) seen in the sick fish A:G ratios. While no conclusive A:G pattern could be ascribed to the sick fish, there does not appear to be an obvious immunosuppressive (low globulin concentration) trend. The globulin protein concentration was statistically different between the 08April (mean 0.73) and 16July (mean 0.57) groups (lower A:G value indicating an elevation in globulin proteins). Perhaps a water quality stress induced an acute phase protein response. Electrophoretic analysis of plasma from moribund fish in 1997 showed an increase in the presumptive acute phase protein band, beta 2 (Appendix 3).

Plasma glucose was significantly lower in fish captured after the 16July sample but was not abnormally low (Table 4 and Figure 5). A similar range of glucose values was reported for carp and tilapia (Hughes et al. 1997, Chen et al. 2003). A stress-induced hyperglycemia depends on adequate liver glycogen stores that may have been reduced later in the summer. The unknown degree of handling stress for each fish complicates plasma glucose interpretation.

Chloride values declined after 08April with moribund having the lowest values (Table 4 and Figure 6). As a group, the sick fish chloride values were quite variable (71% coef. of variation) which probably influenced the lack of statistical difference with other “normal” fish collected after 08April. A normal plasma chloride range for freshwater tilapia is reported to be 119 - 130 mEq / L (Chen et al. 2003). The mean chloride concentration for the 08April group (103 mEq / L) was closest to this range. Sodium was only measured in the 08 April group and was considered somewhat lower than normal (150 – 160 mmol / L for most freshwater fish). Ion loss from the gill, due to extreme handling stress, was the likely cause.

Species-specific comparisons of blood parameters (plasma glucose, chloride, and protein as well as A:G and Lymphocyte: Granulocyte ratios) were examined for the 08April and 16July sample groups. The other groups were excluded from such analysis for the following reasons: 30July group had only 2 SNS, 15August group were all LRS and the fish in the “sick” group were in different states of health. Only plasma chloride was significantly higher in LRS than SNS (ANOVA, $P<0.001$).

Table 4

Mean (Std. deviation) concentrations and values of plasma total protein, albumin, Albumin / globulin ratio (A /G) , glucose, choride, and sodium. Number (n) of samples reported for each group. Statistical differences, among sample groups for any particular sample date, are indicated by different letters (ANOVA, P< 0.05).

Sample Date	Total Protein (g / dL)	Albumin (g / dL)	A / G	Glucose (g / dL)	Chloride mEq / L	sodium mmol/L
08April	4.1 (1.4) a n = 19	1.5 (0.4) n = 19	0.73 (0.39) a n = 19	140 (43) a n=19	103 (8) a n = 19	126 (13) n = 19
16July	4.0 (1.0) a n = 20	1.2 (0.3) n = 20	0.57 (0.67) b n = 20	136 (58) a n= 20	65 (14) b n = 20	ND
30July	3.2 (1.5) a n = 9	1.0 (0.1) n = 8	0.78 (0.51) ab n = 8	79 (24) b n = 8	60 (17) b n = 8	ND
15August	5.3 (1.5) b n = 10	ND	ND	88 (33) b n = 10	71 (17) b n = 10	ND
Moribund 8/15 – 9/5	1.8 (1.3) c n = 13	1.8 (4.4) n = 13	0.59 (0.36) ab n = 13	81 (54) b n = 10	45 (32) b n = 10	ND

ND = not done

Figure 4. Mean concentration of plasma protein (bar = std dev). Letters indicate statistical differences (P<0.05, ANOVA).

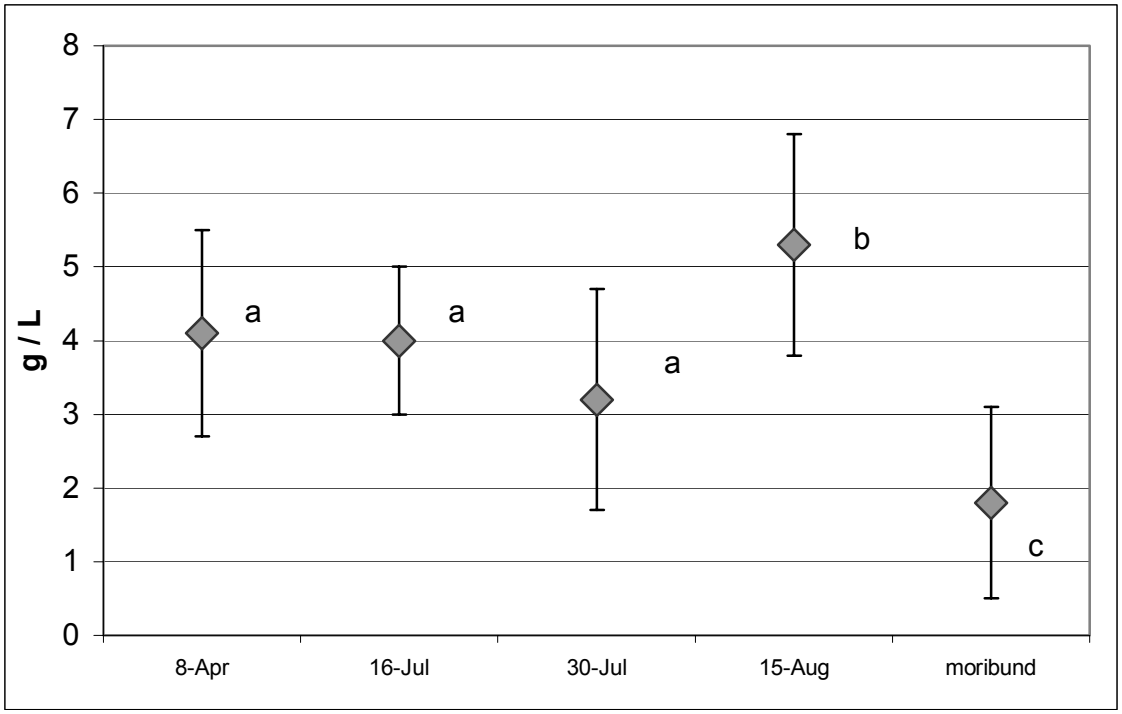


Figure 5. Mean concentrations of plasma glucose (bar = std dev.). Letters indicate statistical differences (P<0.05, ANOVA).

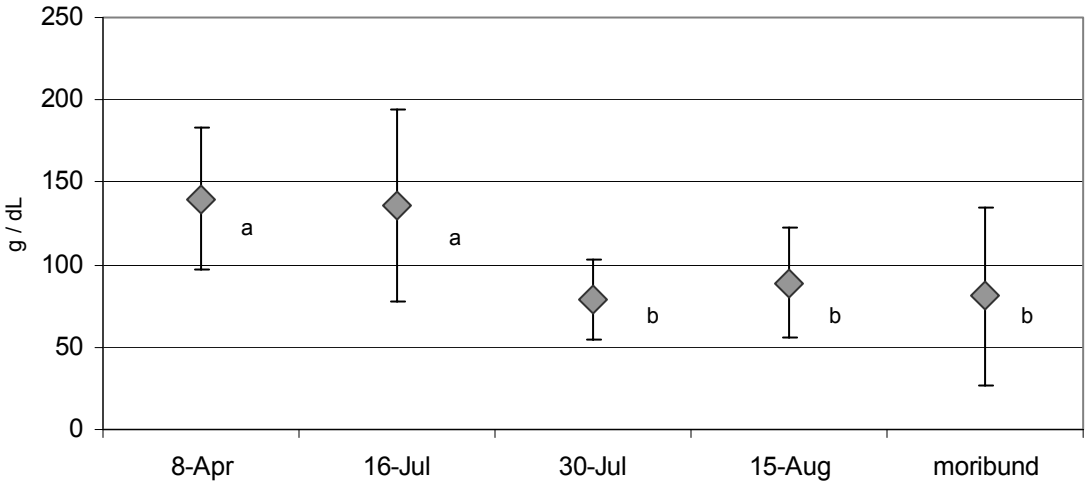
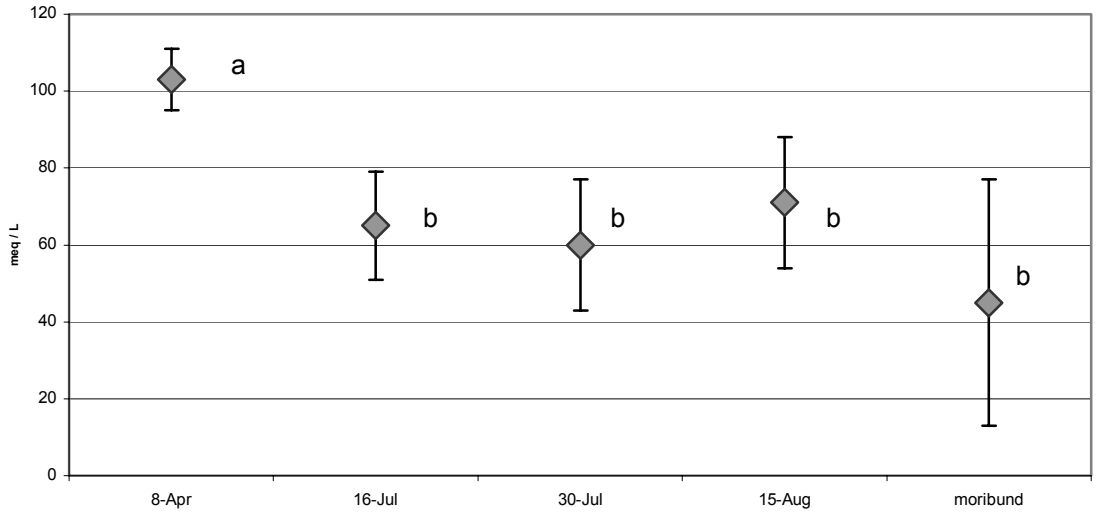


Figure 6. Mean concentration of plasma chloride (bar = std. dev.). Letters indicate statistical differences (P<0.05, ANOVA).

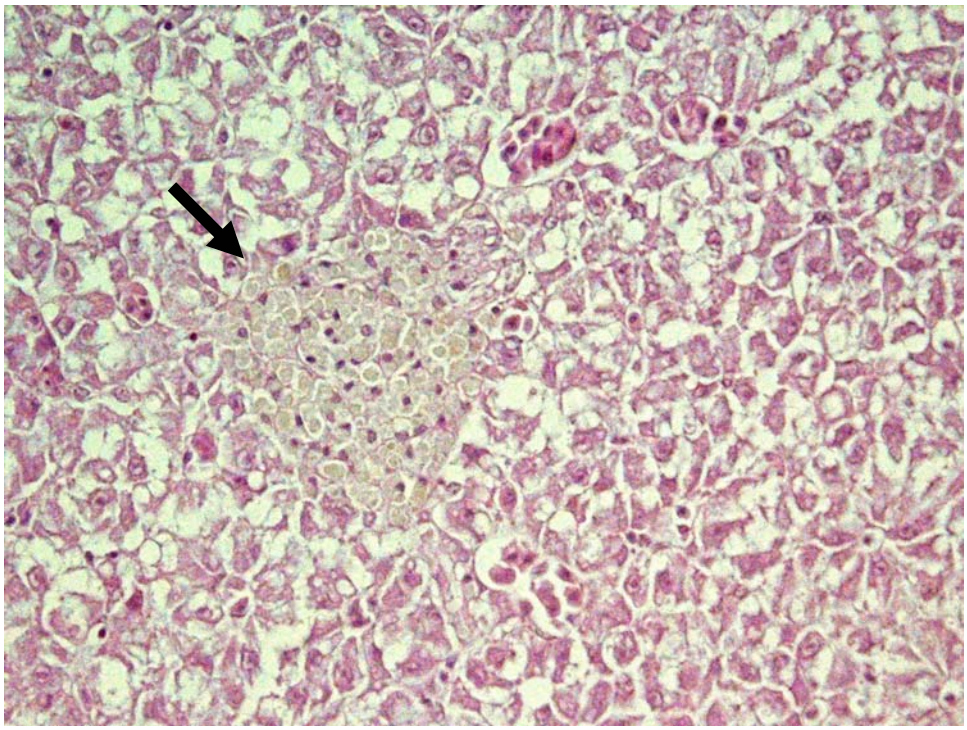


Histology- Sections of gill, kidney, hepatopancreas (liver), and spleen were obtained from 33 suckers. Thirteen of which were identified as sick fish. Gill morphology was quite poor in all specimens due to the extended period exposed to air prior to fixation. A few protozoans (possible *Ichthyophthirius multifiliis*) were observed on the gill of two sick fish (#60 and 62). No significant lesion was associated with the parasites. With the exception of one sick fish (#67), no parasites or significant lesions were observed in any of the hepatopancreas, spleen, or kidney sections. The kidney tubule epithelium of fish 67 contained numerous hyaline droplets that can be associated with ammonia toxicity (Ferguson 1989). Both in 1996 and 1997, hyaline degeneration of the proximal tubule was seen in moribund sucker kidneys (Appendix 3 and 4).

Multiple foci of endogenous brown pigments within macrophages were seen in the kidney, spleen, and hepatopancreas of all specimens (Figure 7). When illuminated at 360 nm, the autofluorescence characteristic of lipofuscin was observed in approximately 50 % of the pigment mass seen in the hepatopancreas of fish 4 (08April). Less than 30% of the splenic pigment content was identified as lipofucsin in the same fish. This finding indicates that varying amounts of both lipofuscin and hemosiderin were present in these pigment foci. A nominal scoring (0, 2,4,6) system for total endogenous pigment within the section showed that the quantity of pigment was relatively low for these organs (median of 2 for all organs, average score 1.1 to 2.7). No obvious difference in pigment quantity was observed between the 3 sample groups (08 April, 16 July, and sick fish). Similar observations of endogenous pigment foci were made for both “normal” suckers collected in May and moribund fish collected in September 1997 (Appendix 3). Lipopigments, such as lipofuscin and ceroid, are polymerized residues of peroxidized lipids and proteins. These membrane bound pigments are usually within secondary lysosomes of the cell (Sohol and Brunk 1989). The insoluble yellow-brown pigment, lipofusion is often referred to as “aging pigment” and is result of oxidative damage to the cell (Cotran et al. 1989). Hemosiderin is a golden-brown pigment composed of aggregates of ferritin micelles and occurs when there is an excess of iron in tissue (Cotran et al 1989). Hemosiderosis in fish is usually associated with either hemolytic anemia or excess dietary / environmental iron intake (Thiyagarajah et al.1998). Low quantities of hemosiderin is typically concentrated in the reticuloendothelial cells of the spleen and kidney as they recycle iron from degraded erythrocytes.

In the 08April sample, the ovary of female #4 was deemed usual in shape and color. The histological section showed the ovary containing eggs in all stages of development and no abnormalities. Foci of blue staining, coccobacilli –shaped forms were observed in the ovary but were not associated with inflammation. It is uncertain whether these forms are an artifact or yeast.

Figure 7. Hepatopancreas section from Snortnose sucker collected near the Williamson River on 2May 2003. Foci of macrophages with endogenous pigments (arrow). Shrinkage of hepatocytes due to fixation artifact. 1000X, Hematoxylin and eosin stain.



Summary Observations:

1. Handling stress complicated interpretation of blood assays. Some sick fish had low plasma protein and chloride concentrations reflective of gill and epidermal lesions. Plasma glucose values tended to be high due to capture stress however 38% of the sampled fish (18 of 48) had values lower than 80 g / d L. These fish may not have had sufficient hepatopancreas glycogen reserves to mount a normal hyperglycemic stress response.
2. Columnaris was the primary pathogen associated with morbidity. Blood – borne aeromonad infections were common and may not be indicative of a disease situation.
3. *Lernaea* infestation was common in all fish collected after 08April. The absence of this copepod in the April sample indicates a seasonality in infectivity or a spatial influence.
4. No obvious sign of immunosuppression (increased A:G ratios, absence of leukocytes) was detected among both “normal” and sick fish.
5. No consistent histological abnormality was observed among either “normal” and sick fish.

Recommendations for future work

1. Determine the relationship of elevated pH changes and fish physiology (stress indicators, blood cell population, immune functions, ion and acid-base regulation) in controlled laboratory studies. See Appendix 2 proposal. Additional water quality parameters (ammonia and dissolved oxygen) can be added in later experiments

2. Examine the temporal aspects of water quality changes associated with algal bloom events and mortality patterns. Do fish die-offs result from multiple events and infection of endemic pathogens (e.g. Columnaris, Lernaea, aeromonads) or is there a chronic loss throughout the summer?

3. Reduce some health sampling effort (histology, gill swab imprints, TYES cultures) that are ineffective. If plasma chemistry analysis is warranted, these measurements should be compared with controlled laboratory studies for better interpretation.

4. Conduct health and energy reserve assessment of juvenile fish in the fall to examine any association with winter kill.

Appendices

1 USGS Field protocol
2 USFWS / USGS Science Support Partnership proposal
3 1997 fish health data, CA-NV FHC and ODFW
4 1996 histological findings of sick fish tissues
5 2003 data

Reference:

Chen Y, GA Wooster,RG Getchell, PR Bowser, and MB Timmons. 2003. Blood chemistry of healthy, nephrocalcinosis-affected and ozone-treated tilapia in a recirculation system, with application of discriminant analysis. Aquaculture 218:89 – 102.

Cotran RS, V. Kumar and SL Robbins. 1989. Robbins Pathologic Basis of Disease. 4th ed., WB Saunders Co., Philadelphia.

Ferguson HW. 1989. Systemic pathogy of fish. Iowa State Press, Ames Iowa.

Hughes GM, T. Szegletes, and J Nemcsok. 1997. A comparison of blood composition in the common carp (*Cyprinus carpio* L.) using samples obtained from caudal vessels and dorsal aorta cannulae. Acta Biologica Hungaria 48(2): 145 – 155.

Jacobs DS, BL Kasten, WR DeMott, WL Wolfson. 1990. Laboratory Test Handbook, 2nd ed., Wiliams and Wilkins, Baltimore.

Modra H, Z Svobodova and J. Kolarova. 1998. Comparison of differential leukocyte counts in fish of economic and indicator importance. Acta Vet. Brno 67: 215 – 226.

Sohol RS and UT Brunk. 1989. Lipofuscin as an indicator of oxidative stress and aging. *In:* Lipofuscin and ceroid pigment, ed.EA Porta, pp17 – 29, Plenum Press New York.

Thiyagarajah A., WR Hartley and A. Abdelghani. 1998. Hepatic hemosiderosis in buffalo fish (*Ictiobus sp*). Mar. Environ. Res. 46(1-5): 203 – 207.

Appendix 1 USGS field protocol for sample collection

A short S.O.P. for collecting samples for adult fish heath monitoring.

1. Identify fish to species and sex.
2. Measure and record fork length and weight.
3. Examine and record external condition – skin and gills.
4. If fish is to be released, then mark with a PIT tag and record number (only PIT tag fish during springtime adult monitoring). If fish is to be sacrificed, then mark with a floy tag and record number.
5. Swab gills between 1st and 2nd arches then streak TYES media in test tube and roll swab across the three windows on the gill swab slide.
6. Collect a little over 1 ml of blood from caudal vein using a syringe (3 ml syringe w/ 21 or 22 gauge needle) that had been previously coated with heparin solution. Drop 1 or 2 drops of blood onto a paper towel to clear needle and for fish to be released place 2 drops of blood into test tube with BHIA media. Place 1 drop of blood on blood smear slide and use another slide to draw blood across to make smear. Fill capillary tube with blood and plug with clay then spin down and measure hematocrit %. Place the remaining blood in capped conical tube and store on ice until you can centrifuge sample. Spin whole blood for about 3 minutes. Use pipettor to collect 3 200-uL aliquots of plasma placing 1 aliquot each in the clear, pink, and blue capped conical tubes. Bag tubes separately by color and place on dry ice.
7. After collecting blood release the fish or if sacrificing fish go to step 8.
8. Place fish in water bath with lethal dose of anesthesia.
9. After fish is fully anesthetized, retrieve fish and using scissors and forceps remove about a 1 cm width of gill tissue (including the arch), dab on a paper towel to remove some of the blood and place in the tube with the histology preservative.
10. Open up the fish using scissors to cut into the body cavity beginning at the vent along the lower side of the fish up to the head. Be sure to lift with the scissors while cutting to avoid damaging the internal organs.
11. Examine and record condition of internal organs.
12. Poke translucent yellow plastic probe into spleen and streak BHIA media in test tube.
13. Cut out small pieces (1 – 1.5 cm^2) of spleen, liver, and kidney and place in the tube with the histology preservative along with the gill tissue. Total amount of all tissues should be no larger than the end of your thumb.
14. If fish is a female, remove and preserve ovaries (eggs skeins) for fecundity work.
15. Remove and prepare opercles and otoliths for age and growth work. If there isn't the time to do this, bag the whole bodies and freeze until there is time to remove and prepare opercles and otoliths. If you don't want to bring back the bodies remove the heads, place them in labeled Ziploc bags, and store them in the freezer until they can be processed.

1. **Identification number**: 04-R1-XX

2. **Title**: Effects of upper Klamath Lake water quality on blood chemistry and immune defenses of adult Shortnose sucker (*Chasmistes brevirostris*).

3. **FWS Project Officer**: Dr. J. Scott Foott, Project Leader, California – Nevada Fish Health Center, 24411 Coleman Hatchery Road, Anderson, CA 96007, phone 530 – 365 – 4271, fax 530 – 365 – 7150, Scott_Foott@fws.gov

4. **USGS Contact**: Scott P. VanderKooi, Fishery Biologist, Klamath Falls Duty Station, 6937 Washburn Way, OR 97603, phone 541-273-8689, fax 541-273-8692, Scott_vanderkooi@usgs.gov

5. **FWS Research Coordinator**: Paul Heimowitz, Aquatic Invasive Species and Research Coordinator, U.S. Fish and Wildlife Service, Region 1, 911 NE 11th Ave, 6E, Portland, OR 97232-4128, phone 503-872-2763, Paul_Heimowitz@fws.gov

6. **Partnerships and roles**: CA-NV Fish Health Center will perform the water quality challenge and laboratory aspects of the project. Contact will be J Scott Foott (see #3).

7. **Type of Support Requested:** Research

8. **Problem Statement and Implications**: Both the Lost River and Shortnose sucker of the upper Klamath basin are listed as endangered species by the USFWS. Poor water quality associated with summer blue-green algal blooms (*Aphanizomenon flos-aquae*) are associated with fish kills and disease outbreaks. During the growth phase of a bloom, pH can rise from 8.5 to 10. Dissolved oxygen (DO) concentrations < 2.0 mg / L and un-ionized ammonia (NH_3-N) levels ranging from 0.2 – 2.0 mg / L. often occur post-bloom due to algal decay. Bioassay work has shown the suckers to be quite tolerant of these adverse water quality conditions with the exception of DO levels < 1.58 mg /L. Despite their resilience, disease outbreaks due to infection with endemic parasitic and bacterial pathogens, is common among the sucker population during the summer months. The role of chronic exposure to adverse water quality on the immune defenses has not been documented. Such information is needed to understand the mechanisms associated with disease outbreaks and accurately model fish survival in relation to specific water quality parameters.

9. **Objectives:**
    a. Determine the duration of exposure to elevated pH and ammonia, typical of Upper Klamath Lake summer algal bloom conditions, that impair non-specific immune functions, normal blood chemistry, and induce histopathological tissue changes in adult Shortnose suckers (SNS).
    b. Year 2 : perform similar work with Lost River suckers, adapt experimental design with data from year 1 work.

Goal: Provide managers with data to model the probability of sucker disease and morbidity in relation to the duration of a blue-green algal bloom and the associated poor water quality.

10. **Methods and study area:** Twenty five adults would be collected in Upper Klamath Lake in May 2003, transported to individual pen areas in two 700 gal. flow-through rearing tanks at the CA-NV FHC wet lab, individually tagged for identification and given prophylactic treatments for external parasites and systemic bacterial infections. The wetlab is a 1152 sq ft facility supplied with ozone-disinfected water that contains various tank configurations for temperature control experimentation and a chlorine effluent disinfection system. Acclimation to base conditions (normoxic, pH 8.5, 22°C, < 0.01 mg/L NH_3-N) would be deemed successful when the majority of fish begin to feed. If pre-experiment mortality is greater than 10% or fish refuse feed for > 18 days, the population will be returned to the lake. Following acclimation, the 10 – 12 fish within the treatment system will be exposed to pH 9.5 water that contains the median June concentration of total ammonia. These conditions will be met over a 3 day ramping period. Controls will remain at acclimation conditions. Blood will be obtained non-lethally from fish at time zero (2 fish), day 15 (4 fish), and day 30 (4 fish) post-treatment in both the treatment and control systems. We plan to avoid repeat bleeding from the same fish. One fish (per group / sample period, n = 6 total) will be lethally sampled for histological tissue examination plus any obviously moribund fish. Blood chemistry assays for cortisol, lysozyme activity, complement activity, chloride, sodium, glucose, hemoglobin, hematocrit, WBC total and differential counts, protein concentration and electrophoresis profile (albumin / globulin ratio), pH, pO2, pCO2, peripheral blood leukocyte phagocytosis activity (inert target and bactericidal activity) will be conducted on each fish to compare treatment with control conditions as well as duration effects. ***The non-lethal blood assays were selected to examine a fish's status for ion balance, stress level, blood cell composition, and non-specific immune function***.

11. Project Duration: January xxx – December 30, 20xx

12. Priority: The USFWS Biological opinion on USBR operations of the Klamath project would benefit from information on the relationship between chronic exposure to elevated pH and ammonia (algal bloom conditions) and sucker health. This information would allow managers to make informed decisions on the probability of chronic disease outbreaks after a given time period of adverse water quality and thereby identify thresholds that would harm the lake's sucker population

13. **Products and schedule:**

1/2004 upon acceptance of proposal, obtain CDFG permission for fish transfer (earlier conversations indicate that support is likely due to the wetlab's effluent disinfection system and the lack of virus isolation from the population.

- **a.** 1/2xx - 5/20xx: Construct and test re-circulation rearing units to control pH, temperature, ammonia, and dissolved oxygen. Perform 30 day holding and feeding trials with Sacramento sucker (surrogate) in test units. Perform assay validation tests on adult Chinook blood samples for several blood measurements (e.g. Radiometer pH,pO2, pCO2). Estimated > 250 hrs labor.
- **b.** 5/20xx – 7/30/20xx: Perform wetlab experiment and return fish to lake estimate >250 hrs labor
- **c.** 8/20xx – 12/ 20xx: Perform clinical laboratory assays, data analysis, report writing (estimate 160 hrs labor). Final product will be a written report to FWS, tribal, ODFW, CDFG, USGS, USBR fishery groups in the Upper Klamath Basin as well as a presentation of results to these cooperators (est. 80 hrs). If appropriate, a manuscript will be submitted to an American Fishery Society journal.
- **d.** 1/20xx –4/20xx: Conduct presentations and obtain input for 20xx experimental design, prepare for 20xxexperiment.
- **e.** 5/ 20xx: Repeat experiment with Lost River sucker adults
- **f.** Similar time frame as 20xx, report by 12/20yy.

14. Budget:

FY04

Operating expenses

Travel	$100	
Vehicle costs	$350	
	Subtotal	$450

Supplies & equipment:

Assay reagent	$2200		
Histology solvents / supplies	$400		
Wetlab chemicals & feed		$400	
pH controlled pumps (4)		$3000	
conditioning tank (1)	$700		
chemical mix tanks (2)		$250	
Radiometer ABL70 blood analyzer	$16000		
		Subtotal	$22950

Salaries:	GS7 technician (240hr)	$4910	
	GS5 biotechician (400hr)	$4500	
		Subtotal	$9410

Total FWS request $32810

In-kind donation:

USGS capture efforts (4 days, 4 person crew, O&M)	$ 2500
FWS (2 biologist 400hrs, lab overhead $1000)	$17100
Total in-kind	$19600

Overhead breakouts:

FWS request		$32810
FWS in-direct 15%		$ 5790
	Subtotal FWS	$38600
USGS WFRC 5% indirect		$ 2030

FY 2004 total request $40630

Budget

FY05

Operating expenses

Travel		$110	
Vehicle costs		$360	
		Subtotal	$470

Supplies & equipment:

Assay reagent	$2670		
Histology solvents / supplies	$450		
Wetlab chemicals & feed		$450	
Replacement heater /chiller	$2100		
		Subtotal	$5670

Salaries:

GS7 technician (240hr)		$5160	
GS5 biotechician (400hr)		$4700	
		Subtotal	$9860

Total FWS request $16000

In-kind donation:

USGS capture efforts (4 days,4 person crew, O&M)	$ 2700
FWS (2 biologist 400hrs, lab overhead $1100)	$18000
Total in-kind	$20700

Overhead breakouts:

FWS request		$16000
FWS in-direct 15%		$ 2800
	Subtotal FWS	$18800
USGS WFRC 5% indirect		$ 1000

FY 2005 total request $19800

15. **Approvals and submittal**:
    a. FWS Project Officer: see attached email

USGS contact:

Appendix 3 memorandum of FWS & ODFW laboratory results of 1997 sampling

UNITED STATES GOVERNMENT **FISH AND WILDLIFE SERVICE**

Memorandum

TO: Mark Buettner / Mike Green BOR
Steve Lewis / John Bower , KF-FWO
Rich Holt, ODF&W

DATE: September 18, 1997

FROM: J. Scott Foott
CA-NV FHC, Anderson, CA

SUBJECT: Diagnostic laboratory results - Klamath Lake suckers

Summary: Adult suckers were examined in May ("healthy") and during a die-off in September. Infection with common aquatic bacteria and parasites appear to be the primary cause of morbidity in the sick fish. Both groups had signs of cellular oxidative damage (lipofuscin pigment), however, it is unclear whether this is due to environmental conditions or a normal aging effect in these fish. The infected fish demonstrated a normal immune response.

In response to high mortality of endangered Short-nosed (SNS) and Lost-river suckers (LRS) in Klamath lake, the FHC attended an interagency meeting in Klamath Falls on January 8, 1997. Poor environmental conditions have been associated with the bacterial and parasitic infections observed in the sick fish (suckers, chubs, trout). A protocol for fish health sampling was developed with Dr. Rich Holt (ODF&W) and on May 20, 1997 health and physiological evaluations were performed on post-spawn suckers collected in the Williamson River (northern Klamath Lake). These samples were to represent "healthy" fish for later comparison with sick animals. In August and September 1997, a fish kill episode occurred in the lake. Samples from moribund suckers were sent to the ODF&W pathology laboratory and the FHC for analysis. The results of work completed at the CA-NV Fish Health Center are discussed below. Station funds were used to cover all FHC expenses.

<u>May 20 Samples</u>
Six SNS and 1 LRS were collected by trammel nets during the morning and examined by Drs. Tony Amandi and Craig Banner (ODF&W) for bacteria and macroparasites. I have spoken with Dr. Holt (ODFW) and was told that the bacterial assays were inconclusive due to probable contaminates associated with windy conditions, however, common aquatic bacteria were isolated from the fish. One sucker (04954) had *Lernaea* sp. parasites (copepod) and a columnaris lesion on its gill. Trematodes were seen inside several fish.

An organosomatic examination as well as blood and histological sampling was conducted by myself. Six of 7 fish had some degree of erythema (minute hemorrhages in the skin along the underside of the fish) and fin erosion. It was unclear if the netting operation produced these abnormalities. All of these post-spawn fish had a moderate

amount of visceral fat which indicated sufficient energy reserves. Six juvenile LRS (mean FL = 108 mm) from the tribal hatchery were also sampled for blood and histological samples. All the juveniles appeared healthy.

Sept 10 - moribund fish sample

Mike Green (BOR-K. Falls) electro-fished near the mouths of 2 creeks on Sept 10 to collect sick fish. Past experience had shown that sick suckers move to cleaner water area (springs) yet were still difficult to catch by only a hand-net . He captured 6 SNS, 1 LRS, and 1 Rainbow trout (data of the trout not included below). Blood and histological samples were sent by overnight delivery to FHC. The blood samples arrived in good condition and were immediately processed for hematological and serological tests. Mike reported that 5 of the 7 suckers had columnaris lesions in the gill as well as *Lernaea* infestation. In late August, moribund chub and suckers were shipped alive to the ODFW fish pathology lab in Corvallis. Dr. Holt informed me on 8/26/97 that these fish had a high prevalence of Columnaris lesions (external infection by *F. columnare)* and severe *Lernaea* infestation.

May - Sept. Sample Comparisons

Organosomatic and disease data- The suckers examined in both collections were of similar size and had "normal" condition factors (table 1). Common aquatic bacteria and parasites were found in fish from both samples (Sept10 blood samples 7 of 7 had high concentrations of *Aeromonas hydrophila*). As in the past, *Flavobacterium (Flexibacter) columnare* infections were the most obvious cause of morbidity, however, large numbers of external parasites (leeches, anchor worm or *Lernaea*) also contributed to their diseased state.

Histological results - Kidney, liver (hepatopancreas), spleen, gonad, gill, heart, and intestinal tissue sections were examined by light microscopy. Lipofuscin deposits were seen in the spleen, kidney, and to a lesser extend the liver of all adult in **both** samples. The juveniles also had very slight amounts of this pigment in their kidneys. These golden-brown deposits were negative for iron (hemosiderin), generally did not auto fluoresce (ceroid), and were distinct from melanin granules. Lipofuscin is referred to as the "aging pigment" and is formed by the oxidation of cellular lipid into insoluble phospholipid polymers. It is a sign of membrane lipid peroxidation by free radicals. I have also observed similar deposits in healthy adult Sacramento suckers captured in Battle creek (site has good water quality). It is unclear whether the presence or quantity of lipofuscin deposits can be used as an indicator of environmental stress or is a normal occurrence in these adult fish. The Sept10 kidney samples had a high prevalence of renal tubule abnormalities in comparison to the May20 fish. Five of the six Sept10 fish had some tubule degeneration and extensive hyaline deposition in the renal epithelial cells of the distal segment. This lesion suggests abnormal levels of protein in the nephron filtrate. No other significant abnormalities or signs of inflammation were observed in the other tissues. Two gill parasites were seen in the May20 suckers; miracidia (*Sanguincola*) in fish =tag 04958 and a cyst of presumptive *Myxobolus* sp. spores were found in the gill of fish (tag 04955). Another May20 sucker had a nematode infection of its visceral fat.

If the lipofuscin deposits are discounted, no definitive signs of toxicity or infection were identified in the sampled fish.

Blood assays - As seen in table 1, the number of red and white blood cells per volume of blood declined in the Sept10 sample group (as measured by hematocrit and Leukocrit). As mentioned above, bacteria were isolated from the blood of the moribund suckers collected on Sept10 and this septicemia had a marked effect on the white blood cell composition. Differential counts of leukocytes revealed a large increase (14 - 70 % compared with 8 - 10 % in healthy fish) in the number of blood phagocytes of Sept10 fish. Many of these activated monocytes contained bacteria in their phagosomes. There was a corresponding drop in the number lymphocytes in these sick fish. Both total protein content and the osmolarity of plasma was lower in the sick Sept10 fish in comparison with the "healthier" May10 suckers. This data is probably related to the columnaris tissue damage. The healthy juvenile suckers had the highest plasma osmolarity (mean 284 $\pm$ 1 mOsm). I would consider this osmolarity value a better "normal" than the May20 fish as it corresponds with data from healthy chinook smolts in freshwater. While the albumin : globulin ratio was similar for the two groups, the sick fish tended to have elevated Beta fractions in electrophoresis preparations (Table 2). Acute phase proteins associated with infection are found in the Beta fractions. As noted by the high coefficient of variation, there was great deal of individual variation. Overall, the Sept10 fish demonstrated that they were responding to the septicemia and had sufficient liver function to produce plasma proteins.

A number of metabolite, enzyme, and ion assays were run on the May10 plasma samples and the data is listed in an attached table. I do not have any experience with non-salmonid plasma values but did notice that the alkaline phosphatase (ALKP u/l) appeared quite high for fish. This enzyme is released from damaged liver cells and is usually < 20 u/l for healthy adult chinook. The glucose and triglyceride values look normal and back up the visceral fat observations. It appears that May20 sample group had adequate energy reserves and could respond to handling stress. We may run the Sept10 plasma samples in the future.

Please distribute this information to other interested parties as appropriate.

Attachments: Organosomatic data sheets (3)
May10 clinical chemistry data

Email
cc: Wayne White, CA Supv. (Without attachments)
Cindy Barry, RO - ES
R. Iverson, KR-FWO
B. Halstead, CC-FWO

Table 1. Organosomatic and clinical chemistry data. Mean (± SEM) and coefficient of variation (%) reported for suckers collected on May 20 (Williamson R.) and September 10, 1997 (Upper Klamath Lake - near Odessa and Harriman creek).

	n = 7 **May20**		n = 7 **Sept10**	
Fork Length (mm)	396 (± 20)	13%	398 (± 30)	20%
Weight (g)	801 (± 103)	34%	802 (± 143)	47%
Condition Factor $(Wt / FL^3) \times 10^5$	1.27 (± .05)	10 %	1.24 (± .08)	18%
Hematocrit (%)	44 (± 2)	10 %	34 (± 5)	37%
Leukocrit (%)	1.36 (± .09)	18%	0.85 (± .08)	25%
Plasma protein (g/dL)+	2.7 (± 1.0)	56%	1.9 (± 0.3)	35%
Plasma Osmolarity (mOsm)	278 (± 5)	5%	236 (± 7)	7%
Albumin / Globulin ratio	0.55 (± .11)	48 %	0.50 (± .05)	27%

\+ BCA microplate method.

Table 2. Plasma protein electrophoresis data. Mean percent area (± SEM) and coefficient of variation (%) of select bands. Identification of select band based on work with chinook juveniles. The Sept10 blood samples were sent in chilled heparinized tubes and were assayed approximately 24 hrs post-collection. The May20 samples were frozen on site.

	n = 7 **May20**		n = 7 **Sept10**	
Pre-albumin	23 (± 3)	28%	15 (± 3)	49%
Albumin	26 (± 3)	28%	27 (± 2)	15%
Globulin 2 & 3 **(alpha)**	30 (± 2)	19%	24 (± 3)	30%
Globulin 4 & 5 **(beta)**	16 (± 3)	41%	25 (± 2)	18%
Globulin 6 **(gamma)**	5 (±1)	62%	7 (± 2)	80%

OREGON DEPARTMENT OF FISH AND WILDLIFE FISH EXAM FORM

SOURCE: Upper Klamath Lake

EXAM DATE: August 22, 1997 **COMPLETION DATE:** 9/4/97

LOT: **Report Number** RH97-147

SPECIES: **Suckers, chubs,** marbled sculpin, redband trout

SIZE:

PONDS:

REASON FOR EXAM

Inspection **Abnormal Loss** X **Preliberation** **Routine**

Other:

SIGNS OF DISEASE:

On August 20, a USGS-BDR crew surveyed the lower portion of Upper Klamath Lake and found dying suckers and chubs. Mark Buettner of Bur. of Reclamation indicated 43 suckers were collected and thousands of chubs were observed in an area south of Eagle Ridge. On August 22, Mike Green and others of the BOR collected dying and dead fish in the lake near Pelican Bay and Rocky Point and in Short, Odessa and Harriman creeks. They also collected fish from the lower end of Upper Klamath Lake at the "A" Canal Diversion. The moribund fish were transferred in water in a transport tank and the dead fish over ice to Oakridge, Oregon where John Kaufman, ODFW, transferred the fish to other containers and delivered the fish to the Corvallis Fish Pathology Lab. There were 14 dying and 15 dead fish from the upper area of the lake and 11 moribund fish from the diversion canal submitted. Fish included 14 adult shortnose suckers, 1 adult Lost River sucker, 12 blue chub, 9 Tui chub, 3 marbled sculpins, and 1 rainbow trout. Thanks to Dave Simon, OSU, for the fish identification. Findings for each fish are listed on the attached table. Many fish had classic columnaris gill lesions and many had extremely high numbers of copepods (*Lernaea)* (more than we have ever seen) attached on the dorsal skin and base of the fins of the adult suckers. The large rainbow trout and the chubs also had much *Lernaea* . Dissolved oxygen levels are 2-5 mg/l and water temperatures exceed 22°C in many areas of the lake.

RESULTS:

Classic columnaris outbreak along with severe copepod infestation. *Fl. columnare* was recovered from 32 (80%) of 40 and Lernaea was found on 29 (72.5%) of 40 fish. Other things also found were external fungi and opportunistic bacteria (aeromonads pseudomonads) commonly associated with columnaris outbreaks. A heart trematode, identified by Dr. Bob Olson, OSU as a *Cotylurus* sp was found in the heart of the suckers. A few leeches were found attached (much less than in 1996). *Myxobolus sp.* in the spleen of the one sucker. *Ichthyobodo* and *Gyrodactylus* also found. No viruses were isolated (see attached form).

RECOMMENDATIONS:

As in 1996, there may be a multitude of severe environmental conditions including high water temperatures, low oxygen, high ammonia levels and other unknown factors involved in causing stress to the fish and causing them to be susceptible to columnaris infection. Perhaps these conditions have also contributed to the incredible bloom of copepods. Also, because it is known that strains of *Fl. columnare* may vary considerably in virulence, there may also be very virulent strains of this bacterial pathogen present. If we have the opportunity we will examine the virulence of strains we have isolated.

Date: 12/19/97 **Pathologist:** R. Holt, C. Banner, J. Kaufman, T. Amandi, M. Engelking

Healthy 0 **Hatch: Upper Klamath Lake**

Moribund 25 **LOT:**

Dead 15 **SPECIES:** Suckers, chubs **Report** RH97-147

MICROSCOPIC EXAM:

Classic columnar stacks typical of columnaris disease were observed when wet mounts of gill, mouth or nare lesions were prepared.
One shortnose sucker had a tremendous number of *Ichthyobodo* (*Costia*) on the gills.
Myxobolus spores (somewhat deformed) were found in the spleen or gall bladder of two shortnose suckers.

CULTURE EXAM: **Media used: TYES + Skim milk, cytophaga agar**

Fish Cultured: 40 **Tissue: Kidney, gill, mouth, spleen, nares**

Flavobacterium columnare colonies were isolated from 32 of 40 fish.
Opportunistic bacteria such as aeromonads and pseudomonads were also present in gills or kidney of all fish examined.

Fl. columnare was isolated from 21 of 28 gills cultured and from 22 of 40 kidney cultures.

Species	Sample No. fish	No. fish with Fl. columnare isolated	No. fish from which Lernaea observed
shortnose sucker	14	10	14
Lost River sucker	1	0	1
Blue chub	12	10	7
Tui chub	9	8	6
Marbled sculpin	3	3	0
Redband trout	1	1	1
	40	32 (80%)	29 (72.5%)

SIGNIFICANT COMMENTS:
Attached summary sheets of results are included.

EIBS **BKD** **ERM** 0 40 **CWD** 0 40

FUR 0 40

Copies: M. Buettner, M. Green, BOR, R. Smith, R. Berry,ODFW, Scott Foott, USFWS

UNITED STATES GOVERNMENT **FISH AND WILDLIFE SERVICE**

Memorandum

TO: Mark Buettner, BOR- Klamath Falls DATE: October 2, 1996

FROM: J. Scott Foott

SUBJECT: Results of histological examination of sucker tissues Case 96-96

Summary: Lesions characteristic of bacterial infections were seen in 14 of 15 fish submitted for examination. All the fish showed hydropic degeneration of a specific region of the renal tubule indicative of a toxin.

Fixed tissues from the 12 moribund and 3 apparently normal shortnose or Lost River suckers , you collected on 05 and 06SEP96 , arrived at the FHC on 11SEP96 and were processed into 0.5 um sections stained with hematoxylin and eosin.

Gill - Degenerative changes such as severe epithelial hyperplasia, focal necrosis, hyperemia, and foci of hemosiderin pigment were seen in 9 of the 15 gill sections. Epithelial separation along the secondary lamellae was common to all sections and could be an artifact of fixation timing or processing. Fungi and bacteria were observed in several of the sections.

Intestine - Seven of the 15 fish (including 1 "normal" fish) showed degenerative changes in the intestine such as inflammatory cell infiltration of the lamina propria, erosion of the epithelium, and lipofuscin deposit. Bacteria were observed in one fish with a necrotic intestine.

Liver- Degenerative changes (some of which are reversible) such as vacuolation and cloudy swelling of the hepatocyte cytoplasm, focal zones of liquefactive necrosis, and periportal infiltration of bile ducts was seen in 14 of the 15 fish. Bacteria were seen in the blood vessels of 8 of these livers.

Kidney - The most significant lesion seen in this organ was the hyaline droplets or complete hydropic degeneration of the tubule epithelial cells. This degenerative change was only seen in one region of the nephron (second proximal tubule). This part of the nephron is involved in ion and glucose transport. Most of the kidneys contained interstitial foci of brown pigment (not melanin) and necrotic cells with this same brown pigment. I believe that this pigment may be either lipofuscin (phospholipid breakdown product) and / or hemosiderin (hemoglobin breakdown product indicative of hemolysis). This brown pigment is probably not ceroid as it did

not autofluoresce.

Conclusions

These fish, including several of the "normal" fish, were systemically infected with bacteria and showed lesions characteristic of such infections. I could not specifically identify the species of bacteria nor did I observe filamentous bacteria associated with the necrotic gills. It is not unusual for external columnaris disease lesions to be colonized by aquatic bacteria and fungus . This phenomena often obscures detection of the filamentous bacteria such as *Flavobacterium columnare (*formally *Flexibacter columnaris*).

The specific lesion observed in the kidney is indicative of toxic tubular necrosis which can be caused by heavy metals, pesticides, and other poisons. I do not have any specific information about the effects of the blue-green algal toxin, microcystin, on the kidney, however, there is a report describing that gill sodium-potassium-Adenosine Triphosphatase (Na-K-ATPase) pump is inhibited by microcystin. The second proximal tubule would also contain this type of ion pump(s) and could be similarly affected by microcystin.

In order to answer the questions about the effect of environmental stressors and infectious agents on these fish, it would be necessary to perform a prolonged health survey of the population and bioassays with the suspected toxins.

Please contact me if you have any questions.

cc: J. Grover , DARD -R1
R. Holt, ODFW- Corvallis
R. Garrett, Klamath ERO

Appendix 5 2003 database
c:krtr03\UKLsucker03
Upper Klamath Lake Sucker Health - 21003
old lamprey marks common

0=NONE
1=<10%

2x5p viral 4/8case=0CPE **2=>10-30%OR HEMOR**

FAX DIFFICULT TO READfor necropsy data

fish	samID	samdate	species	SEX	SICK	floy	pit	SITE	EPI-LES
1	1	8-Apr	LRS	F	N	4570		MODOC PT	1
2	2	8-Apr	SNS	F	N	4256		MODOC PT	1
3	3	8-Apr	LRS	M	N	4257		MODOC PT	1
4	4	8-Apr	LRS	F	Y	4567		MODOC PT	0
5	5	8-Apr	LRS	F	N	4568		MODOC PT	1
6	6	8-Apr	SNS	F	N	4569		MODOC PT	1
7	7	8-Apr	SNS	F	N	4571		MODOC PT	1
8	8	8-Apr	LRS	M	N	4566		MODOC PT	1
9	9	8-Apr	SNS	M	N	4255		MODOC PT	1
10	10	8-Apr	SNS	M	N	4254		MODOC PT	1
11	11	8-Apr	SNS	F	N		132672446A	MODOC PT	1
12	12	8-Apr	LRS	F	N		A651295630	MODOC PT	1
13	13	8-Apr	LRS	F	N		132671446A	MODOC PT	2
14	14	8-Apr	SNS	M	N		A053031513	MODOC PT	1
15	15	8-Apr	LRS	M	N		A051083815	MODOC PT	1
16	16	8-Apr	SNS	F	N		132712?5?A	MODOC PT	1
17	17	8-Apr	SNS	F	N		331?4671A	MODOC PT	1
18	18	8-Apr	LRS	M	N		132709494A	MODOC PT	1
19	19	8-Apr	SNS	M	N		32726232A	MODOC PT	1
20	20	8-Apr	LRS	F	N		A052777554	MODOC PT	1
73		2-May	SNS	F	??			Williamson R.	
21	21	16-Jul	SNS	F	N			BALL PT	0
22	22	16-Jul	SNS	F	N			BALL PT	1
23	23	16-Jul	SNS	m	N			BALL PT	0
24	24	16-Jul	SNS	F	N			BALL PT	1
25	25	16-Jul	SNS	F	N			mouth WilliamsonR	1
26	26	16-Jul	SNS	F	N			mouth WilliamsonR	1
27	27	16-Jul	SNS	M	N			fish banks	0
28	28	16-Jul	LRS	M	N			fish banks	0
29	29	16-Jul	LRS	M	N			fish banks	1
30	30	16-Jul	LRS	M	N			fish banks	1
31	31	16-Jul	SNS	F	N			fish banks	0
32	32	16-Jul	SNS	F	N			fish banks	1
33	33	16-Jul	LRS	F	N			fish banks	1
34	34	16-Jul	LRS	F	N			fish banks	2
35	35	16-Jul	SNS	M	N			fish banks	0
36	36	16-Jul	SNS	F	N			fish banks	0
37	37	16-Jul	SNS	F	N			fish banks	0
38	38	16-Jul	SNS	M	N			fish banks	0
39	39	16-Jul	SNS	F	N			fish banks	1
40	40	30-Jul	LRS	F	N			Mouth Pelican Bay	0
41	41	30-Jul	LRS	F	N			Mouth Pelican Bay	1
42	42	30-Jul	LRS	F	N			Mouth Pelican Bay	2
43	43	30-Jul	LRS	F	N			Mouth Pelican Bay	1
44	44	30-Jul	LRS	M	N			Mouth Pelican Bay	2
45	45	30-Jul	LRS	F	N			Mouth Pelican Bay	2
46	46	30-Jul	LRS	F	N			Mouth Pelican Bay	2
47	47	30-Jul	SNS	F	N			Mouth Pelican Bay	2
48	48	30-Jul	LRS	F	N			Mouth Pelican Bay	2
49	49	30-Jul	SNS	F	N			Mouth Pelican Bay	2
50	50	13-Aug	LRS	F	N			Mouth Pelican Bay	1
51	51	13-Aug	LRS	M	N			Mouth Pelican Bay	1

52	52	13-Aug	LRS	F	N		Mouth Pelican Bay		1	
53	53	13-Aug	LRS	M	N		Mouth Pelican Bay		2	
54	54	13-Aug	LRS	M	N		Mouth Pelican Bay		2	
55	55	13-Aug	LRS	M	N		Mouth Pelican Bay		2	
56	56	13-Aug	LRS	F	N		Mouth Pelican Bay		2	
57	57	13-Aug	LRS	F	N	A046829012	Mouth Pelican Bay		1	
58	58	13-Aug	LRS	F	N		Mouth Pelican Bay		2	
59	59	13-Aug	LRS	M	N		Mouth Pelican Bay		1	
60	60	15-Aug	SNS	M	Y		Rocky Pnt/ Pelican Bay		2	
61	61	19-Aug	LRS	F	Y		Rocky Pnt/ Pelican Bay		3	
62	62	22-Aug	SNS	F	Y		Pelican Bay/Rocky Point		1	
63	63	27-Aug	LRS	F	Y		Pelican Bay/Rocky Point		0	
64	64	27-Aug	SNS	F	Y		Pelican Bay/Rocky Point		0	
65	65	2-Sep	SNS	F	Y		PelicanBay/Harriman Crk		3	
66	66	2-Sep	SNS	M	Y		PelicanBay/Harriman Crk		1	
67	67	2-Sep	LRS	F	Y		PelicanBay/Harriman Crk		1	
68	68	2-Sep	LRS	F	Y		PelicanBay/Harriman Crk		1	
69	69	2-Sep	SNS	F	Y		PelicanBay/Harriman Crk		0	
70	70	5-Sep	LRS	M	Y		Pelican Bay	2-gillFc		
71	71	5-Sep	SNS	F	Y		Pelican Bay	2-gillFc		
72	72	5-Sep	LRS	M	Y		Pelican Bay	2-gillFc		

Appendix 6 bloodsmear database

MAX STRESS

HCT	Fish# bs#	RBC PARA	diffcnt lym	thromb	neutro	eosino	monoc	total	L:G	blood notes
29	1 y		47	50	2	1	0	100	15.7	low WBC#
33	2	0	70	26	3	0	1	100	23.3	
35	3	0	70	26	1	3	0	100	17.5	
12	4	0	8		2			10		anemic,highPE
29	5	0	57	36	6	1	0	100	8.1	
41	6 y		81	14	5	0	0	100	16.2	
37	7 y		66	31	3	0	0	100	22.0	
24	8	0	53	33	10	4	0	100	3.8	
38	9	0	77	16	7	0	0	100	11.0	
41	10	0	84	12	4	0	0	100	21.0	
44	11 y		92	7	1	0	0	100	92.0	
40	12 y		71	22	7	0	0	100	10.1	
26	13 y		80	15	5	0	0	100	16.0	
40	14	0	89	10	1	0	0	100	89.0	
30	15	0	79	13	8	0	0	100	9.9	
33	16	0	64	24	12	0	0	100	5.3	lowWBC#
34	17	0	77	18	5	0	0	100	15.4	
24	18	0	73	23	4	0	0	100	18.3	highPE
49	19 y		87	8	4	1	0	100	17.4	
39	20 y		47	20	32	0	1	100	1.5	high PE/neutrophilia
	21	0	65	24	11	0	0	100	5.9	
	22	0	18	35	18	1	0	72	0.9	
	23	0	34	16	22	0	0	72	1.5	
	24	0	62	21	17	0	0	100	3.6	
	25	0	31	26	41	2	0	100	0.7	
	26	0	24	55	20	0	1	100	1.2	
	27	0	33	26	41	0	0	100	0.8	
	28	0	55	24	19	0	1	99	2.9	
	29	0								
	30	0	38	32	27	3	0	100	1.3	
	31	0	31	34	35	0	0	100	0.9	
	32	0	33	34	33	0	0	100	1.0	
	33	0	32	52	14	0	2	100	2.3	
	34	0	44	43	13	0	0	100	3.4	
	35	0	59	21	20	0	0	100	3.0	
	36	0	46	33	19	1	1	100	2.3	
	37	0	55	27	18	0	0	100	3.1	
	38	0	42	36	22	0	0	100	1.9	
	39	0	50	45	5	0	0	100	10.0	
	40	0	46	36	18	0	0	100	2.6	

41	0	77	9	12	2	0	100	5.5	
42	0	67	23	10	0	0	100	6.7	
43	0	55	29	13	3	0	100	3.4	
44	0	41	26	33	0	0	100	1.2	
45	0	66	15	19	0	0	100	3.5	
46	0	75	15	10	0	0	100	7.5	
47	0	59	26	15	0	0	100	3.9	
48	0	72	16	12	0	0	100	6.0	
49	0	41	38	21	0	0	100	2.0	
50	0	90	8	4	0	0	102	22.5	
51	0	82	14	2	2	0	100	20.5	
52	0	79	13	6	0	0	98	13.2	
53	0	74	21	5	0	0	100	14.8	
54	0	87	6	8	0	0	101	10.9	
55									unreadable
56									unreadable
57									unreadable
58	0	65	23	10	0	0	98	6.5	
59	0	91	3	4	0	0	98	22.8	
60	0	10	3	89	0	0	102	0.1	
61									unreadable
62	0	10	26	64	0	0	100	0.2	
63	0	20	22	58	0	0	100	0.3	
64	0	52	44	3	0	1	100	17.3	large lymphs
65	0	52	33	15	0	0	100	3.5	large lymphs
66	0	27	20	10	25	18	100	0.8	
67	0	60	11	12	0	17	100	5.0	anemic,highWBC
68	0	45	37	17	0	1	100	2.6	
69	0	60	23	15	0	2	100	4.0	
70	0	72	25	2	0	1	100	36.0	anemic,highWBC
71	0	44	18	6	0	32	100	7.3	
72	0	70	18	9	0	3	100	7.8	

Appendix 7 Bacterial and clinical chemistry data

							3reps					
			gillswab		tyes		Plasma	Plasma	Plasma	Plasma	Plasma	Plasma
BHI#	BHI-tis	bacte	slide#	FilGNR	tyes#	Fc	#	Cl	GLU	TP	ALB	A/G
1	s		1	N	1	N	1	101		1.9	1.1	1.185
2	s		2	N	2	N	2	97		4.0	1.6	0.649
3	s		3	N	3	N	3	115		5.6	1.7	0.43
4	s	Ah	4	N	4	N	4	118		1.2	0.5	0.716
5	s		5	N	5	N	5	109		4.1	1.8	0.768
6	s		6	N	6	N	6	96		3.2	1.8	1.314
7	s		7	N	7	N	7	98		4.8	2.0	0.722
8	s		8	N	8	N	8	108		3.1	1.8	1.376
9	s		9	N	9	N	9					
10	s		10	N	10	N	10	107		3.3	1.4	0.723
11	b		11	N	11	N	11	101		5.1	2.0	0.626
12	b		12	N	12	N	12	107		5.4	2.1	0.627
13	b		13	N	13	N	13	110		2.5	1.6	1.722
14	b		14	N	14	N	14	98		5.5	1.7	0.459
15	b		15	N	15	N	15	101		4.7	1.2	0.346
16	b		16	N	16	N	16	95		3.1	1.1	0.577
17	b		17	N	17	N	17	88		5.3	1.6	0.427
18	b		18	N	18	N	18	112		4.1	1.4	0.514
19	b		19	N	19	N	19	97		6.4	1.6	0.344
20	b	Pfluor	20	N	20	N	20	102		4.6	1.3	0.404
21	b	AP	21	N	21	N	21	60	78	5.5	1.58886	0.406
22	b		22	N	22	N	22	87	138	3.7	0.7371	0.249
23	b		23	N	23	N	23	65	94	4.2	1.25689	0.427
24	b		24	N	24	N	24	46	73	2.4	0.91619	0.617
25	b		25	N	25	N	25	66	147	4.4	1.11275	0.339
26	b		26	N	26	N	26	47	53	2.1	1.61507	3.331
27	b	AP	27	N	27	N	27	49	141	5.1	1.58886	0.453
28	b		28	N	28	N	28	72	239	4.6	0.90308	0.244
29	b		29	N	29	N	29	75	263	3.5	1.27873	0.576
30	b	AP	30	N	30	N	30	70	67	4.6	1.18701	0.348
31	b		31	N	31	N	31	69	151	5.4	1.30931	0.32
32	b		32	N	32	N	32	48	117	2.2	0.8594	0.641
33	b		33	N	33	N	33	74	101	5.5	1.01229	0.226
34	b		34	N	34	N	34	87	161	3.8	0.79825	0.266
35	b		35	N	35	N	35	57	122	3.6	0.90308	0.335
36	b		36	N	36	N	36	63	188	3.7	1.01665	0.379
37	b		37	N		nd	37	74	125	4.7	1.5015	0.469
38	b		38	N		nd	38	50	200	4.1	1.29621	0.462
39	b		39	N		nd	39	53	182	2.7	1.10401	0.692
40	b		40	N	40	N	40	88	71	4.3	1.75921	0.692

41	b	micrococcus	41	N	41	N	41	63	98	3.5	0.89872	0.18
42	b		42	N	42	N	42	89	69	5.9	1.18701	0.62
43	b		43	N	43	N	43	60	69	3.1	0.87251	1.654
44	b		44	N	44	N	44	69	59	1	1.08654	0.338
45	b		45	N	45	N	45	71	62	4.3	0.91619	0.931
46	b	AP	46	N	46	N	46	40	77	1.9	1.17827	0.355
47	b	micrococcus	47	N	47	N	47	42	68	4.5	1.17827	0.829
48	b	AP	48	N	48	N	48	44	132	2.6	0.90745	1.31
49	b	AP	49	N	49	Y	49			1.6		
50	b		50	N	50	N	50	51	91	5.7		
51	b		51	N	51	N	51	56	137	5.9		
52	b		52	N	52	N	52	67	84	4.3		
53	b		53	N	53	N	53	42	43	1.6		
54	b	staphloc	54	N	54	N	54	77	109	6.1		
55	b		55	N	55	N	55	75	64	4.7		
56	b	Ah	56	N	56	N	56	76	132	5.2		
57	b	AP	57	N	57	N	57	79	39	5.6		
58	b		58	N	58	N	58	83	99	6.9		
59	b	AP	59	N	59	N	59	100	90	6.6		
60	b	AP	60	Y	60	Y	60	0	37	0.4	0.2	0.640
61	b		61	lost	61	Y	61			2.0	1.0	1.099
62	b	AP	62	N	62	N	62	31	109	3.4	0.9	0.338
63	b		63	N	63	N	63	21	103	3.4	0.9	0.358
64	b		64	N	64	N	64	100	131	3.1	1.0	0.500
65	b	Ah	65	N	65	N	65	11	25	0.5	0.2	0.690
66	b	Ah	66	N	66	N	66	53	105	1.0	0.3	0.386
67	b	AP	67	Y	67	N	67	75	105	0.2	0.1	0.905
68	b	AP	68	Y	68	N	68	63	26	2.1	0.5	0.324
69	b		69	N	69	N	69	73	5	4.1	0.9	0.262
70	b	AP	70	Y	70	N	70	24	165	1.3	0.5	0.732
71	b		71	Y	71	N	71			1.8	0.9	1.037
72	b	AP	72	N	72	N	72			0.3	0.3	16.471

Appendix 8 histological examination data

Lipofuscin area estimates

0- none, 2 -<or=10%, 4- 11-30%, 6 - >30%

pmc= postmorteum change

histo section#	KD note	KD %LPF	KD para	KD note	LV %LPF	LV para	LV note	SPL %LPF	SPL para	SPL note	Gill	Gill para
4264	Fish1	2	0	glyc vacuoles	2	0		4	0	pmc-poor		0
4265	2	4	0	glyc vacuoles	2	0		4	0	pmc-poor		0
4266	3	2	0	glyc vacuoles	0	0		2	0	pmc-poor		0
4267&68 = fish4		4	0	360nmFA~50%	2	0	melanin10%,hemosiderin50%,LPF<30%	200%	0	pmc-poor		0
4269	5	4	0		2	0		4	0	pmc-poor		0
4270	6	2	0		2	0		2	0	pmc-poor		0
4271	7	2	0	pmc	2	0		2	0	pmc-poor		0
4272	8	2	0		0	0		2	0	pmc-poor		0
4273	9	2	0		0	0		2	0	pmc-poor		0
4274	10	2	0	odd bile duct	0	0		2	0	pmc-poor		0

02 May Williamson R.												
4326	case=03-98,no KD		photo,fix shrinkage		2	0	muscularis portion of intestine?			nd		

21	2	0	0	0	4	0 pmc	0
22	2	0	2	0	4	0 pmc	0
23	2	0	2	0	2	0 pmc	0
24	4	0	2	0	2	0 pmc	0
25	2	0	2	0	4	0 pmc	0
26	2	0	2	0	4	0 pmc	0
27	2	0	0	0	2	0 pmc	0
28	2	0	2	0	2	0 pmc	0
29	4	0	2	0	4	0 pmc	0
30	4	0	0	0	4	0 pmc	0

60	2	0	0	0	2	0	protozoan?	1
61	2	0	0	0	4	0		0
62	2	0	2	0	2	0	ich? HP	1
63	2	0	0	0	2	0		0
64	2	0	2	0	2	0		0
65	4	0	2	0	2	0		0
66	4	0	2	0	4	0		0
67	hyalinedroplet/tub	0	0	0	0	0	0	0
68	0	0	0	0	2	0		0
69	2	0	2	0	2	0	HP no para	0
70	2	0	0	0	2	0	HPw/Necrosis	0
71	2	0	0	0	4	0		0
72	mod. interst. HP	2	0	0	0	2	0	0